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14. ABSTRACT
To enhance in vivo efficacy of anti-EGFR full length IgG for treatment of basal breast cancers, the most aggressive subtypes, we have successfully employed molecular evolution approach to improve Fc binding affinity to Fc receptor that activates ADCC effect in our 2008 report. In the current report, we focus on the molecular evolution of Fc binding affinity to neonatal Fc receptor (FcRn), which modulates the half-life of IgG in vivo. In detail, we first expressed and purified soluble human FcRn in mammalian CHO expression system, then used human FcRn to select yeast-displayed library of Fc domain variants with random mutations. Several Fc domain variants were identified to bind human FcRn with enhanced affinity than wild-type Fc. We also found that by alternating FcRn and FcγRIIIa/V158 in sorting, some variants exhibited higher binding affinity to both human FcRn and FcγRIIIa/V158, which was not observed in previous studies improving the affinity to FcRn while losing the binding to FcγRIIIa/V158. The Fc variants with improved affinity to both FcRn and FcγRIIIa/V158 may show enhanced ADCC and prolonged half-life in vivo.

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Fc domain, Fc receptor, affinity, yeast display

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INTRODUCTION:

The basal phenotype of breast cancers currently are associated with poor prognosis, partially due to the fact that there are no targeted therapies that are directed towards this type of cancer. While epidermal growth factor receptor (EGFR) is frequently overexpressed on basal type breast cancers, the response rate is not dramatic to anti-EGFR antibody therapy. One mechanism by which antibodies in general cause cancer cell death is by activating the immune system via the Fc portion of the antibody. Such activation is called antibody dependent cellular cytotoxicity (ADCC) and has been shown to be important in the anti-cancer activity of some antibodies. To determine the importance of ADCC in the response of basal breast cancers to antibody therapy, and to develop a better basal breast cancer therapeutic, we sought to develop anti-basal breast cancer antibodies with enhanced ADCC

To target basal subtype breast cancers with poor prognosis, we proposed to engineer anti-EGFR full length IgG with enhanced ADCC effect and optimized pharmacokinetics. To achieve this research goal, we designed to improve the affinity of Fc for activating Fc receptors and neonatal Fc receptor using our established yeast display platform. The designed full length IgG will have a previously optimized antigen-binding fragment (Fab) and an improved constant fragment (Fc) that will be identified in this project. We anticipate that the platform generated for enhancing ADCC will work for the evolution of antibody affinity to other Fc receptors, which play important roles in modulating antibody function *in vivo*.

BODY:

Engineering anti-EGFR antibodies for treatment of breast cancers with poor prognosis
Studies and Results

Tasks 1 to 3 have largely been completed and were reported in the first year annual report.

Task 4. To select Fc mutants with increased binding to sFcRn by fluorescent activated cell sort (FACS) (Months 10-13):

a. The displayed Fc mutant library was subjected to 4 rounds of selection using decreasing concentration of sFcRn for staining of yeast.
b. Screen individual clones from 4th round output for increased binding to sFcRn as determined by flow cytometry.
c. Determine the binding affinities of selected clones to sFcRn by flow cytometric analysis on yeast surface.
d. Identify selected Fc mutants by DNA sequencing.

A. A transient 293-system was used to express both human Fc gamma receptors and FcRn, but failed to produce enough FcRn for purification. We then turned to a stable CHO expression system, which we use to express full-length IgG, and successfully produced and purified human FcRn with GST fused to the C-terminal of the α domain and His tagged at the C-end of β domain. The purity of the recombinant human FcRn is over 90% after GSTrap column purification.

In our previous work to improve binding of Fc to FcγRIIIa/V158, the human Fc variants with improved affinity to FcγRIIIa/V158 also bound murine FcγRIV with higher affinity, thus we decided to continue optimizing human Fc, instead of murine Fc, for better in vivo pharmacokinetics of IgGs in vivo in mice.

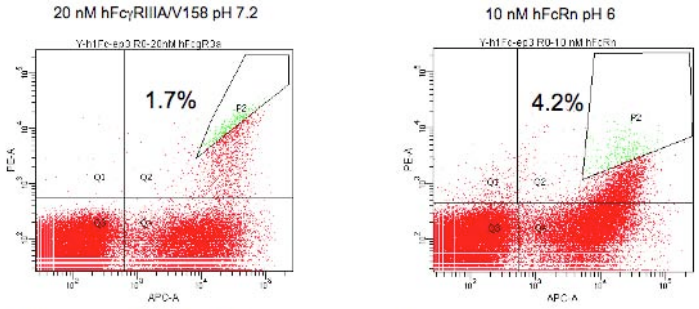


Figure 1. Binding of human Fc variant library to Fc receptors.

The yeast display library of mutant human Fc comprising 2×10^7 variants was subjected to a series of FACS sortings, which alternated human FcRn at pH 6, and FcγRIIIa/V158 at pH 7.2. The staining of human Fc variant library showed that 4.2% of the total yeast cells bound FcRn of 10 nM at pH 6, and 1.7% bound FcγRIIIa/V158 of 20 nM at pH 7.2 (Figure 1). The binding of 1.7% of total yeast cells to FcγRIIIa/V158 at 20 nM was much stronger than that stained with 200 nM FcγRIIIa/V158 described in our last report. The difference in binding signal and affinity may due to the dimerization of Fc receptors via the GST domain. As such, the affinity should always be measured with wild type Fc as control, and considered as a relative value when GST fused soluble receptor is used.

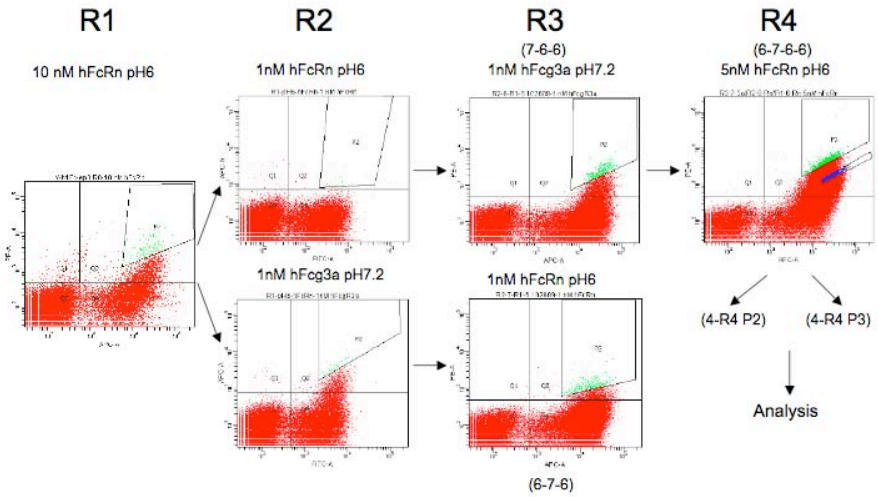


Figure 2. FACS sorting of human IgG1 hinge-Fc fragments with increased binding to human FcRn at pH 6 and human FcgR3a/V158 at pH 7.2.

To select Fc variant with higher affinity for both FcRn and FcγRIIIa/V158, the concentration of Fc receptor was decreased, and also FcRn and FcγRIIIa/V158 were alternated in each round of sorting (Figure 2). In the last round of sorting with FcRn, two

populations showing different binding strength were isolated for analysis.

In total, we completed four FACS sort selections applying different strategies that differed by using different Fc receptors and conditions in each round of selection (Table 1).

Table 1. The sorting strategies:

Sort Strategy	R1	R2	R3	R4
A	50nM hFcRn pH 7	25nM hFcRn pH 7	5nM hFcgR3a pH 7	
B	20nM hFcgR3a pH 7	1nM hFcgR3a pH 7	1nM hFcRn pH 6	
C	10nM hFcRn pH 6	1M hFcRn pH 6	1nM hFcgR3a pH 7	5nM hFcRn pH 6
D	10nM hFcRn pH 6	1nM hFcgR3a pH 7	1nM hFcRn pH 6	

B. Individual yeast clones were picked, grown, induced, and screened for binding to both human FcRn and FcγRIIIa/V158 (Figure 3). Among all the clones screened from strategy “C”, those binding to FcRn at pH 6 also bound FcγRIIIa/V158 at pH 7.2 strongly, indicating that the sorting approach successfully enriched variants binding to both receptors. However, clones isolated from selection strategy “A” (Table 1), which didn’t include FcRn binding at pH 6, failed to bind FcRn with similar strength as that bind to FcγRIIIa/V158. As a result, the Fc domain with desired binding ability to different Fc receptors can be enriched by manipulating the sorting strategy as we demonstrated here.

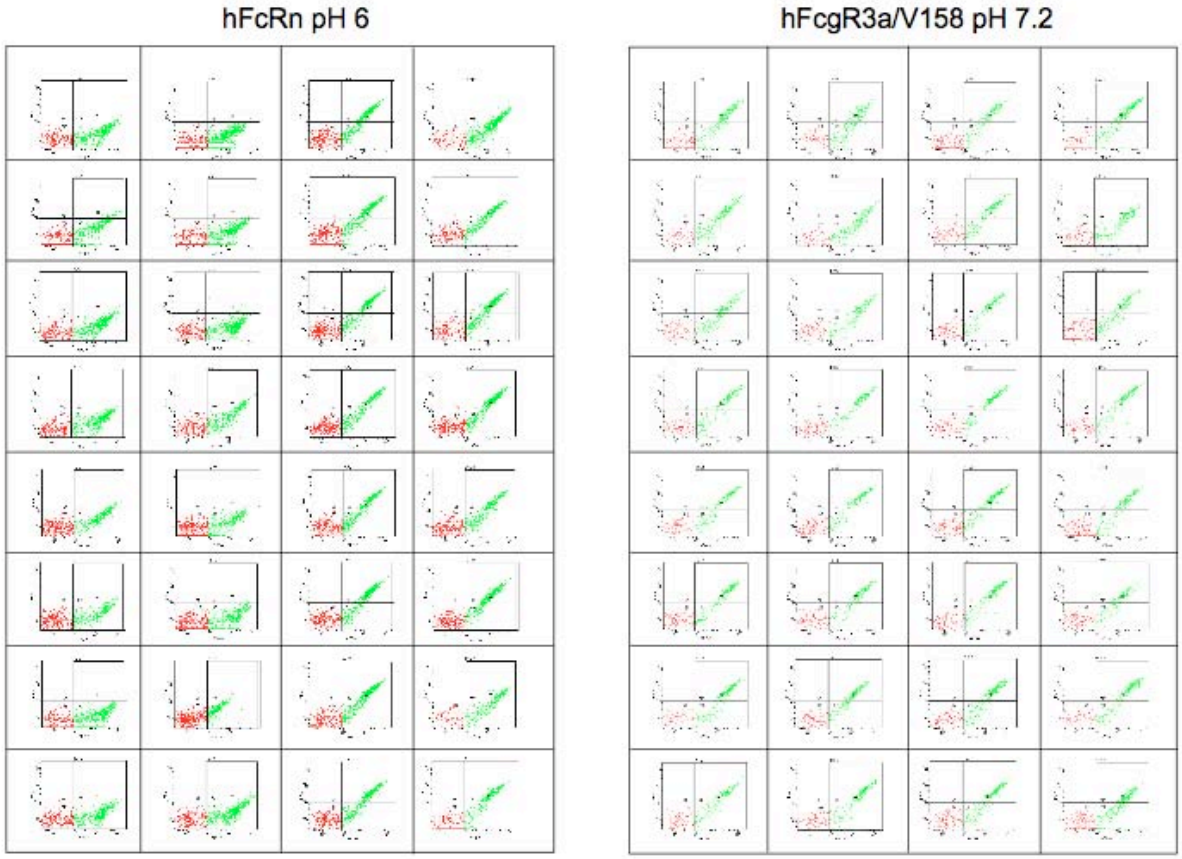
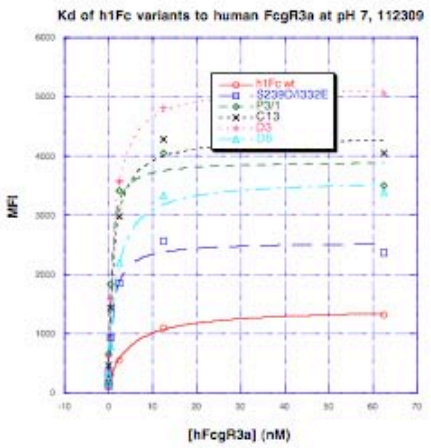


Figure 3. High throughput screening of human Fc variants with differential binding to human FcRn at pH 6 and human FcgR3a/V158 at pH 7.2.

C. The equilibrium dissociation constant (K_D) for human sFcRn and sFcγRIIIA/V158 was determined using flow cytometry (Razai, et.al., 2005) and the best mutant showed seven fold improvement for sFcRn and four fold highr affinity for sFcγRIIIA/V158 compared to wild-type Fc region displayed on yeast surface (Figure 4).

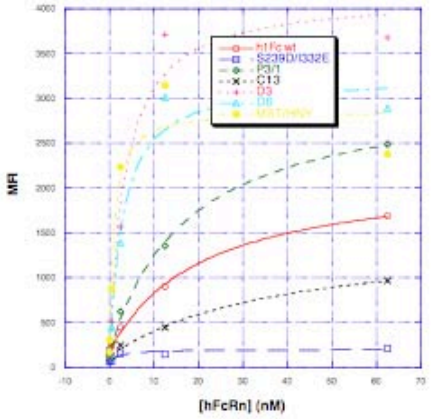
(a)



(b)

hFcgR3a pH 7	KD (nM)
h1Fc wt	5.05
S239D/I332E	0.95
P3/1	0.53
C13	1.11
D3	1.23
D6	1.72

(c)



(d)

hFcRn pH 6	KD (nM)
h1Fc wt	20.6
S239D/I332E	N.D.
P3/1	17.42
C13	37.43
D3	3.39
D6	2.89
MST/HNY	0.91

Figure 4. Binding affinity of yeast displayed human Fc and variants to human Fc receptors.

In previous Fc engineering studies, variant with improved affinity towards one Fc receptor intended to lose binding to the other. Human Fc variant MST/HNY (Sally, 2001) bound human FcRn at pH 6 with the highest affinity of 0.91 nM compared to wild-type Fc of 20.6 nM as determined in our measurement, but failed to bind sFcγRIIIA/V158 (data not shown). Another human Fc variant S239D/I332E (Lazar, 2006) had an affinity 5-fold better than wild-type Fc towards sFcγRIIIA/V158, also showed mere binding to sFcRn. The Fc variant P3/1 isolated by consecutive sorting with sFcγRIIIA/V158 in our last report bound sFcγRIIIA/V158 with 9-fold higher affinity than wild-type Fc, remained similar binding affinity to sFcRn. Only variants isolated by alternating human FcRn at pH 6 and sFcγRIIIA/V158 at pH 7.2 showed improvement in binding to both Fc receptors.

D. The DNA sequencing analysis revealed some hot spots on CH3 for binding to both sFcRn and sFcγRIIIA/V158, which are different from the known regions. Further comparison of all the mutant sequences may facilitate engineering superb Fc domain to develop the most potent IgG antibodies.

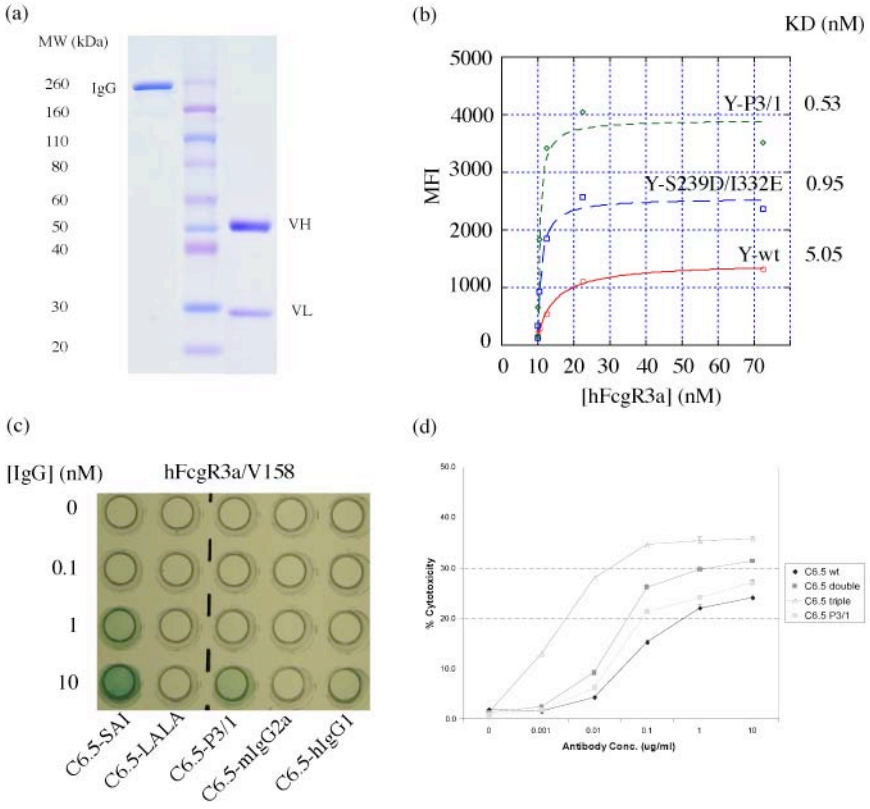
Task 5. To express and characterize a panel of chimeric anti-EGFR IgG1 in a stable CHO expression system (Months 14-18):

a. Subclone the selected murine Fc mutants from Task 3 and Task 4 into an anti-EGFR IgG expression vector.
b. Generate anti-EGFR chimeric IgG1 (murine) in CHO system using readily developed protocols in our laboratory.
c. Determine the binding affinities of soluble anti-EGFR chimeric IgG1 to sFcγRIII, sFcγRIIB and sFcRn by BIAcore, and to EGFR by flow cytometric analysis on EGFR overexpressing cells.

A-B. The gene of the identified human Fc variant was extracted from pYD2 vector by PCR amplification to clone into a mammalian expression vector harboring VH and VL of anti-EGFR antibody or anti-HER2 antibody. After sequencing verification, the expression plasmids were introduced into CHO cells by electroporation, followed by G418 selection and clonal expansion, the IgG protein will be secreted into the culture media and purified by protein-G column with the purity over 90% (Figure 5a).

C. Binding to target antigen, EGFR or HER2, by recombinant human IgG antibodies with Fc variants, was determined by flow cytometric analysis and showed no difference between IgGs consisting of the same VH and VL (data not shown).

The binding analysis of Fc variants displayed on yeast surface identified multiple clones with improved affinity to soluble receptor FcRn and FcγR3a/V158 (Figure 4). However, the binding of IgG with Fc variant P3/1 to FcγR3a/V158 immobilized on an ELISA well showed minimal improvement compared to IgG with wild-type Fc domain (Figure 5c), though the yeast displayed variant P3/1 bound to soluble FcγR3a/V158 with about 10-fold improvement in affinity than that of wild-type Fc domain (Figure 5b).



We further analyzed the ADCC activity of human PBMCs mediated by anti-HER2 IgG C6.5 with a series of Fc variants which showed improved cytotoxic effect for IgG with P3/1 than that with wild-type Fc domain, but less toxic than IgG with S239D/I332E variant (Figure 5d).

Discussion: We have noticed that the binding affinity of yeast displayed Fc domain correlated with the KD measured by other means. However, the affinity variant P3/1 isolated by yeast display molecular evolution resulted in a slightly improvement in ADCC effect than wild-type Fc. It seems that the KD measured on yeast surface may not always correlate with the KD of Fc domain for Fc receptors when Fc receptors are anchored on the cell surface. The difference in binding KD of yeast displayed Fc domain from the recombinant IgG towards Fc receptors may also result from the disparate composition of glycosylation. As such, we will include an analysis step using Fc receptor coated ELISA assay to further screen yeast displayed Fc variants before converted into full-length IgG antibodies.

Task 6. To evaluate the in vivo efficacy of anti-EGFR chimeric IgG1 antibodies generated in Task 5 in xenograft with basal-like breast cancer cell lines (Months 19-24):

a. 10^7 basal-like beast cancer cell line MDAMB468 with high EGFR receptor density (1-5 million) was injected subcutaneously in nude mice for initial evaluation. Ten mice will be included for each study.
b. Three anti-EGFR IgG1 antibodies with the differential binding ratio to sFcγRIII versus sFcγRIIB administered intravenously at a dose of 1mg per mouse based on current protocol at UCSF preclinical core facility.
c. Tumor growth was recorded as standard procedure.

We have not started in vivo studies yet because the in vitro ADCC analyses didn't show improvement for the previous identified Fc variants, such as P3/1. We anticipate accomplishing this task in the coming year.

KEY RESEARCH ACCOMPLISHMENTS:

1. We expressed and purified human FcRn, which is not commercially available.
2. We are able to improve the Fc binding affinity to FcRn at different pH using yeast display.
3. Several Fc variants were selected for the enhanced binding to both human FcRn and human FcγR3a/V158, affinity measured on yeast surface and compared with known Fc mutants.
4. Recombinant full-length IgG antibodies targeting EGFR and HER2 with Fc variant P3/1 isolated from this study, and Fc variant S239D/I332E and S239D/I332E/A330L designed based on 3D structure, were generated using a stable CHO expression system, and analyzed for ADCC activity.

REPORTABLE OUTCOMES

1. Presentation:

Yu Zhou, James D Marks. (2008) Engineering anti-EGFR antibodies for treatment of breast cancers with poor prognosis. Era of Hope, Baltimore, MD, June 25-27, 2008

2. Manuscripts:

Yu Zhou, Yong Tang, Louis M Weiner, James D Marks. (2009) Molecular evolution of Fc affinity for enhanced ADCC (In preparation)

CONCLUSION:

1. Using a stable CHO stable expression system, human FcRn receptor was expressed and purified
2. The binding of both human and murine Fc region to human FcRn receptor was determined on the yeast surface.
3. A human Fc random mutant library was generated, sorted, and individual variants characterized for increased binding affinity to human FcRn receptor.
4. By alternating Fc receptors used for FACS sorting, Fc domain variants with improved affinity for multiple Fc receptors can be generated.

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